Research Article

Genistein- and daidzein 7-O-β-D-glucuronic acid retain the ability to inhibit copper-mediated lipid oxidation of low density lipoprotein

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Two isoflavones *in vivo* metabolites, genistein-7-*O*-β-D-glucuronic acid (G7G) and daidzein-7-*O*-β-D-glucuronic acid (D7G) were synthesised chemically. The ability of these metabolites to scavenge an organic radical was measured by the trolox equivalent antioxidant capacity (TEAC) assay, while their reducing ability was measured by the ferric reducing antioxidant power (FRAP) assay. The TEAC and FRAP values of G7G were 45 and 51% of that of genistein, while those of D7G were 52 and 77% of that of daidzein, respectively. A direct assessment of G7G and D7G antioxidant activity by their ability to delay copper(II)-mediated lipid oxidation of human LDL showed that these metabolites retained the ability to prevent oxidation in the lipid phase, but activity was diminished compared to their corresponding aglycones. However, G7G and D7G also decreased the rate of lipid oxidation to 53 and 86% of control without isoflavones, respectively, indicating a continuous exchange of antioxidants between the aqueous environment and the LDL lipid phase during the whole oxidation period.

Keywords: Antioxidant activity / Daidzein glucuronide / Genistein glucuronide / Isoflavones metabolites / Lipid oxidation

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1 Introduction

Nutritional antioxidants are exogenous antioxidants which are provided by our diet and might be important in the prevention of diseases [1]. Soy beans and soy products constitute a significant part of the Asian diet and are an abundant source of isoflavones, a class of polyphenolic flavonoid antioxidants. The basic isoflavone structure consists of a phenolic B-ring linked to the C3-position of a pyrane's C-ring (Fig. 1). The most abundant isoflavones found in soy are the glucosides, acetyl-glycosides and malonyl glucosides of genistein and daidzein [2, 3]. Genistin and daidzin are the 7-*O*-β-D-glucoside forms the aglycones (Fig. 1). Upon consumption, a small percentage of the glucosides can be directly absorbed through the gastrointestinal tract [4, 5], but most are hydrolysed by intestinal β-glycosidases to yield the corresponding aglycones which are more readily absorbed [6, 7]. Some isoflavones are also metabolised by the colon microflora to form products such as dihydrodaidzein, dihydrogenistein, *O*-desmethylangolensin, 6′-hydroxyl-*O*-desmethylangolensin and equol which are also absorbed. The aglycones are biotransformed by Phase II metabolic processes during their passage through the epithelial cells of the small intestine and the liver to form mainly monoglucuronide plus some diglucuronide, mono- and disulphate and mixed glucuronide–sulphate conjugates prior to their release into the general circulation [8–10]. This conversion apparently increases the polarity of the metabolites leading to their excretion by the kidneys. Nonetheless, these metabolites remain in the circulation for several hours after a meal rich in isoflavones [11], which provide longer plasma exposure compared to other flavonoids such as flavonols and flavanols [12, 13].

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Abbreviations: ABTS, 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid; **D7G,** daidzein-7-*O*-β-D-glucuronic acid; **FRAP,** ferric reducing antioxidant power; **G7G,** genistein-7-*O*-β-D-glucuronic acid; **TEAC,** trolox equivalent antioxidant capacity; **TPTZ,** 2,4,6-tripyridyl-*s*-triazine

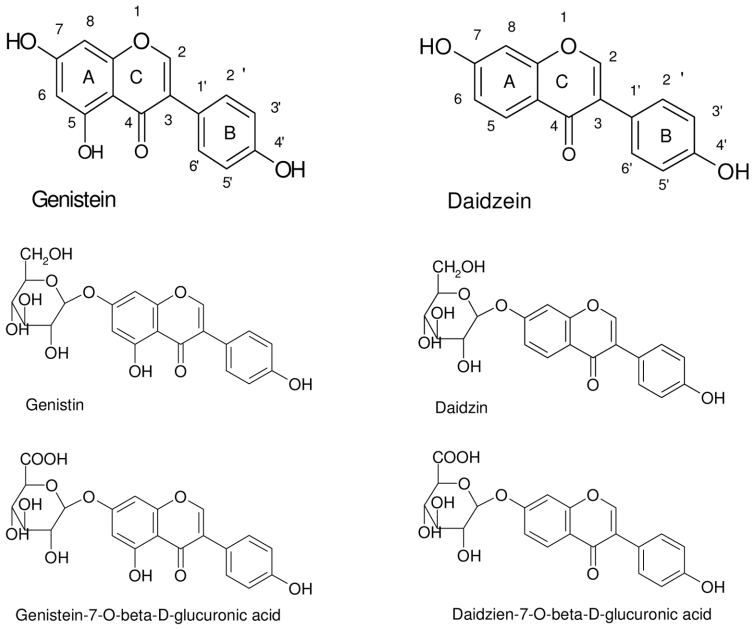

Figure 1. Structures of some naturally occurring isoflavones and *in vivo* isoflavone glucuronide metabolites.

Isoflavones have been shown to possess indirect antioxidant activity as electron/hydrogen donor in chemical reactions [14 – 18] and direct antioxidant activity as lipid radicals scavenger by inhibiting iron or copper-mediated lipid oxidation in LDL and liposomal systems [17 – 24]. Evidence from studies linking consumption of soy with protection of LDL against oxidative modification in human [25 – 29] and animal [30] models seems to indicate that biologically relevant isoflavone metabolites retain their antioxidant activity *in vivo*. The ability of isoflavones to inhibit the lipid oxidation chain reaction would require the isoflavones partition into the lipid phase of LDL vesicles where the chain reactions occur. Indeed, both genistein and daidzein have been shown to be able to partition into LDL vesicles [16, 17, 31, 32]. However, the ability of their glycosides, genistin and daidzin, to partition into the lipid phase was reduced and this reduction was also correlated to a reduction in their capacity to inhibit LDL oxidation [18]. No information was available for the biologically relevant and apparently more polar glucuronide metabolites. These more water-soluble isoflavone metabolites might not partition into lipid phase of LDL vesicles, but they may still be able to exert their antioxidant activity through recycling of vitamin E radical in these vesicles.

In this paper, we report for the first time the antioxidant activity of genistein-7-*O*-β-D-glucuronic acid (G7G) and daidzein-7-*O*-β-D-glucuronic acid (D7G), the predominant *in vivo* metabolites from soy consumption [9, 10, 13] by measuring their trolox equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP), and their ability to directly inhibit copper(II)-mediated lipid oxidation of human LDL.

2 Materials and methods

2.1 Chemicals

Acetobromo-D-glucuronic acid methylester, 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), daidzein, daidzin, DMF (anhydrous), EDTA (disodium salt), $FeCl_3 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, genistein, genistin, hexadeuterodimethyl sulphoxide (DMSO-d_6), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®), LDL (low density lipoprotein from human plasma, lyophilised powder), potassium carbonate (anhydrous), tetramethylsilane and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were obtained from Sigma – Aldrich (St. Louis, MO, USA). All other common chemicals and solvents used were of analytical or HPLC grade and water was of Millipore® HPLC Deionised Water grade.

2.2 Equipments and apparatus

UV absorption measurements were performed with a Cary 3 Bio UV – Visible Spectrophotometer (Variant, USA) fitted with a Cary Temperature Controller and UV block cell with a built in water jacket. Rotary evaporation under vacuum was performed with a BUCHI RotaVapor RE111 rotary evaporator fitted with water jet vacuum system and a BUCHI 461 temperature-controlled water bath (Copenha-

gen, Denmark). Equipments for HPLC and LC-ESI-MS are listed under the respective sections.

2.3 Synthesis of G7G and D7G

Genistein- and daidzein-*O*-β-D-glucuronic acids were chemically synthesised using the Koenigs–Knorr reaction. Daidzein (200 mg) or genistein (200 mg) was reacted with acetobromo-α-D-glucuronic acid methylester (1 g) in anhydrous DMF according to the method of Moon *et al*. [33], but with modifications to the purification steps. The acetyl moiety was first removed by treatment with sodium methylate followed by hydrolysis of the methyl ester in alkaline water as described to yield a mixture of deblocked β-*O*-glucuronide products. The solution was acidified with formic acid and solvent removed by rotary evaporation. Twenty millilitres of acidified methanol containing 1% formic acid was added and insoluble materials were removed by filtration using filter paper (Whatman). The filtrate was further filtered using a 5 μm membrane filter disc (Millipore) and then concentrated to 4 mL by rotary evaporation. A sample was analysed by RP HPLC using an analytical C_{18} column (below). Putative genistein-*O*-β-D-glucuronic acid (or daidzein-*O*-β-D-glucuronic acid) peaks were identified by LC-ESI-MS in separate chromatography (below). The major glucuronide peak was purified by RP HPLC using a semipreparative C_{18} column (below). The yield was determined by spectroscopy using molar absorption of 3.90×10^4 L mol^{-1} cm^{-1} for genistein and 2.75×10^4 L mol^{-1} cm^{-1} for daidzein products at λ 260 nm [34]. Proton NMR spectroscopy (below) identified the linkage as the 7-*O*-β-D-glucuronic acid.

2.4 HPLC

The analytical RP HPLC system consisted of an Alltech (USA) Altima™ 3 μm particle size C_{18} silica guard column disc (5 mm length × 7 mm diameter) and main column (53 mm length × 7 mm diameter) connected to a UV detector (Waters™ 490E Programmable Multiwavelength Detector, Milford, MA, USA) and an HPLC separation module (Waters 2690). Data acquisition and manipulation was performed with a Shimadzu (Japan) C-R5A Chromatopac™. The column was developed with a water/ACN gradient (containing 1% acetic acid) from 5 to 50% in ACN at a flow rate of 1 mL/min over a 20 min period, with wavelength absorbance of the UV detector set at 260 nm. The elution times of genistein and daidzein were established using authentic standards.

The semipreparative RP HPLC system consisted of a Phenomenex (USA) Luna™ 10 μm particle size C_{18} guard column (50 × 10 mm) and main column (250 × 10 mm) connected to a UV detector and data acquisition and manipulation as above. The injection volume was 200 μL and the column was developed with a water/ACN gradient (containing 1% acetic acid) from 5 to 40% in ACN at a flow rate of 2.5 mL/min over a 30 min period, with wavelength absorbance of the UV detector set at 260 nm. The column was washed with 100% ACN and equilibrated to starting condition before the next injection. The major putative genistein-*O*-glucuronide or daidzein-*O*-glucuronide peak was collected and pooled from multiple runs, the solvent evaporated by rotary evaporation and compound dissolved in methanol to 500 μM concentration using the molar absorptions of the aglycones.

2.5 LC-ESI-MS

LC-ESI-MS analysis was conducted in positive mode electrospray ionisation on a Waters Micromass quadrupole TOF (Q-TOF) mass spectrometer coupled to a Waters 2795 HPLC. Chromatographic separation was achieved with a Phenomenex Synergi Polar RP C_{18} column (Torrance, CA, USA; 4 μm particle size, 50 mm length × 2.1 mm diameter) maintained at 40°C, and eluted with a water/ACN gradient (containing 0.05% formic acid) at a flow rate of 0.4 mL/min. The ionisation of the flavonols was optimised at a capillary voltage of 3.2 kV and cone voltage of 40 eV. The following MS conditions were maintained during analysis: Desolvation temperature of 350°C, source block temperature of 90°C, desolvation gas flow of 400 L/h and cone gas flow of 100 L/h. MS and MS-MS spectra were collected in the mass range of 100–1000 mass units and CID experiments were conducted with collision energy of 30 eV.

2.6 ^{1}H NMR spectroscopy

Samples recovered from semipreparative HPLC were evaporated to dryness *in vacuo* and redissolved in hexadeuterodimethyl sulphoxide solution (DMSO-d_6). ^{1}H NMR spectra were recorded on a Bruker Avance 300 MHz spectrometer (Bruker, Wissembourg, France) at an operating frequency of 300 MHz. Chemical shifts (δ) were reported relative to tetramethylsilane in ppm.

2.7 Trolox equivalent antioxidant capacity assay

The capacity of isoflavones to scavenge an organic free radical was measured by the TEAC assay and was carried out according to Arts *et al*. [35] with minor modifications. The ABTS$^\bullet$ free radical chromophore was produced by the oxidation of ABTS (7 mM) with potassium persulphate (2.45 mM) in methanol overnight. Before the experiments were performed, the radical was diluted with methanol to give an absorbance of about 0.7 at 734 nm wavelength. Isoflavones were prepared at a range of concentrations in methanol and a 100 μL aliquot sample was mixed with 1 mL of the ABTS$^\bullet$ solution to give final concentrations ranging from 4.5 to 90.9 μM. The absorbance was recorded at 734 nm wavelength after 5 min reaction time at RT. A

standard curve was prepared using different amount of Trolox at final concentrations ranging from 4.5 to 90.9 μM. The TEAC measured using ABTS$^{\bullet}$ represents radical scavenging activity as molar equivalent of Trolox. This was obtained from the ratio of the slopes (= Δ*A per* μM) of the linear plots for an isoflavone and Trolox.

2.8 The FRAP assay

The reducing capacity of isoflavones was measured by the FRAP assay and it was carried out according to Benzie and Strain [36] with minor modifications. The method was based on the direct reduction of Fe(III)–2,4,6-trpyridyl-*s*-triazine complex (Fe(III)–TPTZ) to the Fe(II)–TPTZ form by an antioxidant. Stock solutions of sodium acetate buffer (300 mM, pH 3.6), TPTZ (10 mM in 40 mM HCl) and $FeCl_3 \cdot 6H_2O$ (20 mM) were prepared. TPTZ reagent for the assay was freshly prepared prior to use by mixing 25 mL acetate buffer with 2.5 mL TPTZ and 2.5 mL Fe(III) solutions. Isoflavone sample (100 μL) in methanol was mixed with 1 mL of the TPTZ reagent to give final concentrations ranging from 4.5 to 90.9 μM, and the absorbance of the Fe(II)–TPTZ chromophore was recorded at 593 nm wavelength after 10 min reaction time at RT. A standard curve was prepared using different amount of Fe(II) (as $FeSO_4 \cdot 7H_2O$) in place of Fe(III) in the TPTZ reagent at a final concentrations ranging from 4.5 to 90.9 μM. The FRAP represents the reducing capacity of an antioxidant as molar equivalent of Fe(II). This was obtained from the ratio of the slopes (= Δ*A per* μM) of the linear plots for an isoflavone and Fe(II).

2.9 Inhibition of lipid oxidation

Lyophilised human LDL was reconstituted as a suspension to 500 μg/mL of protein with PBS buffer containing 0.01% EDTA by sonic mixing in a bath sonicator for 10 min at 25°C.

Cu(II)-mediated LDL oxidation was performed at 37°C in a capped 1.5 mL UV quartz cell (1 cm path length, 2 mm width) by the addition of 10 μL of 100 μM $CuSO_4$ to a mixture containing 10 μL methanol, 100 μL LDL in PBS (50 μg protein) and 880 μL PBS. The final reaction concentrations were 1 μM Cu(II), 50 μg protein/mL LDL and 1% methanol in PBS. The PBS was purged with nitrogen for 1 min prior to use to remove dissolved oxygen which may participate in LDL oxidation. The reaction temperature was maintained by a Cary Temperature Controller. The oxidation of LDL was monitored for the formation of oxidised lipid conjugated diene products by absorbance measurement at 234 nm wavelength. Lag time of LDL oxidation was determined as the time coordinate of the intersection of the tangents to the increase in conjugated diene during lag and propagation phases [37] (Fig. 2). Both lag and propagation phase lines were fitted by least square regression. To study the effect of isoflavones or isoflavone glucuronides on the reaction, the methanol addition was replaced by 10 μL of the antioxidant in methanol (500 μM) to give a final antioxidant concentration of 5 μM. The intra-assays CV for increased in lag phase over control was 3.4%.

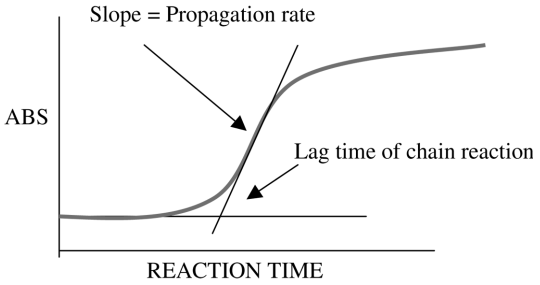

Figure 2. Determination of lag time and propagation rate in lipid oxidation experiment.

The propagation rate is defined as the maximal rate of formation of conjugated diene during the propagation phase and is determined from the slope of the propagation phase. The molar absorbance ε_{234} of conjugated diene is 29 500 L mol^{-1} cm^{-1} and by using this value, the rate of conjugated diene formed *per* litre and minute for a cell of 1 cm can be calculated [38].

$$\text{Propagation rate } (\mu\text{M}) = \frac{\Delta\text{Absorbance}}{\text{Time (min)}} \times \frac{10^6}{29\,500}$$

$$= \frac{\Delta\text{Absorbance}}{\text{Time (min)}} \times 33.8$$

2.10 Statistical analysis

All values are expressed as mean ± SD from triplicate or more reactions. One-way analysis of variance (ANOVA) was used to compare mean lipid oxidation levels as absorbance subjected to the different treatments. Student's *t*-test was used to determine statistical significance. The difference was designated as significant when $p < 0.05$.

3 Results and discussion

3.1 Chemical synthesis of G7G and D7G

Genistein-*O*-β-D-glucuronic acid was chemically synthesised by reaction with acetobromo-α-D-glucuronic acid methyl ester in a Koenigs–Knorr reaction followed by sequential deprotection by removal of the acetyl and methyl groups. The β-configuration arises from anomeric inversion of the reaction. Analytical RP HPLC of the reaction mixture revealed a number of earlier eluting reaction products (Fig. 3a) beside the genistein starting material, which was eluted at 18.483 min. LC-MS-MS identified the major 12.430 min product peak as a genistein monoglucuronide

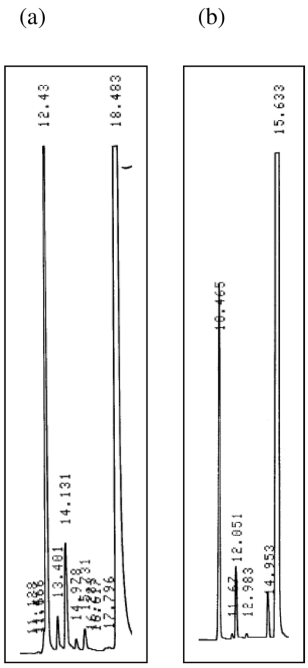

Figure 3. C18 RP HPLC chromatogram of (a) genistein and (b) daidzein glucuronides reaction products.

by its molecular ion ($[MH]^+$) at *m/z* 447.187 and the genistein aglycone fragment (M-glucuronic acid) at *m/z* 271.118 (Fig. 4a). It was purified to greater than 98% homogeneity by semipreparative RP HPLC based on peak area in analytical HPLC. The yield was 14% by mole, calculated from its absorbance at 260 nm wavelength and using the molar absorption of genistein.

Daidzein-*O*-β-D-glucuronic acid was similarly synthesised and the analytical RP HPLC also revealed a number of earlier eluting reaction products (Fig. 3b), with the daidzein starting material eluting at 15.633 min. LC-MS-MS identified the major 10.465 min product peak as a daidzein monoglucuronide by its molecular ion ($[MH]^+$) at *m/z* 431.193 and the daidzein aglycone fragment (M-glucuronic acid) at *m/z* 255.119 (Fig. 4b). This product was also purified to greater than 98% homogeneity by semipreparative RP HPLC with an 8% yield by mole using the molar absorption of daidzein.

The earlier eluting time of daidzein compared to genistein in RP HPLC is well known, despite the apparent higher polarity of genistein due to the presence of an extra C5-OH [17]. Thus, it was also observed that daidzein glucuronide eluted earlier than genistein glucuronide (Fig. 3). This behaviour probably arises from intramolecular hydrogen bonding between the hydrogen of C5-OH and oxygen of C4-keto groups in genistein that decreased the polarity of the molecule.

LC-ESI-MS also indicated that the minor peaks eluted before the starting material of the two reactions were composed of incompletely deprotected monoglucuronides and possibly monoglucuronides of different linkage positions. These by-products were not pursued further as the focus of the study is on the 7-*O*-β-D-glucuronides as these are the biologically relevant and predominant *in vivo* metabolites from soy consumption [9, 10, 13].

Table 1. ^{1}H-NMR spectral data for the chemically synthesised G7G and D7G (δ in DMSO-d_6)

Position	Genistein-7-*O*-β-D-GlcA	Daidzein-7-*O*-β-D-GlcA
H2	8.41 (s)	8.37 (s)
H3	Absent	Absent
C4′-OH	9.60 (s)	9.60 (s)
H5	Absent	8.04 d (9)
C5-OH	12.91 (s)	Absent
H6	6.71 (d, *J* = 2 Hz)	7.13 (d, *J* = 9 Hz)
C7-OH	Absent	Absent
H8	6.47 (d, *J* = 2 Hz)	7.20 (d, *J* = 2 Hz)
H2′,6′	7.38 (d, *J* = 9 Hz)	7.39 (d, *J* = 9 Hz)
H3′,5′	6.82 (d, *J* = 9 Hz)	6.80 (d, *J* = 9 Hz)
GlcA-H1″	5.23 (d, $J_{1'',2''}$ = 7 Hz)	5.22 (d, $J_{1'',2''}$ = 7 Hz)
GlcA-H2″	3.53 (m)	3.53 (m)
GlcA-H3″	3.56 (m)	3.56 (m)
GlcA-H4″	3.68 (m)	3.68 (m)
GlcA-H5″	3.83 (d, *J* = 9 Hz)	3.83 (d, *J* = 9 Hz)

J, coupling constant; s, singlet; d, doublet; m, multiplet; GlcA-H1″ to H5″, glucuronic acid protons.

3.2 ^{1}H NMR

^{1}H NMR spectroscopy identified the linkages of the two products as G7G and D7G (Table 1). The 12.430 min genistein monoglucuronide peak purified by semipreparative HPLC showed proton resonance signals for C4′-OH (9.60 ppm, singlet), C5-OH (12.91 ppm, singlet) and GlcA-H1″ (5.23 ppm, doublet, $J_{1'',2''}$ = 7 Hz) protons and none for C7-OH (expected 10.89 ppm, singlet) proton, consistent with a G7G structure. The 10.465 min daidzein monoglucuronide peak purified by semipreparative HPLC showed proton resonance signals for C4′-OH (9.60 ppm, singlet) and GlcA-H1″ (5.22 ppm, doublet, $J_{1'',2''}$ = 7 Hz) protons but none for C5-OH and C7-OH protons, consistent with a D7G structure. The coupling constant for the anomeric proton of the glucuronide moiety ($J_{1'',2''}$ = 7 Hz) for both products was characteristic of the β-anomer.

3.3 Radical scavenging antioxidant activity

The radical scavenging antioxidant activity of G7G and D7G were measured using the TEAC assay (Table 2) and their activities compared to other isoflavones. This assay measures an antioxidant capacity to scavenge an organic radical ($ABTS^{\bullet}$) by its ability to donate an electron or a hydrogen atom to the radical. Theoretical investigations have suggested that both the C5-hydroxyl (present in genistein) and C7-hydroxyl (present in both genistein and daidzein) are not important in antioxidant activity [39]. In fact,

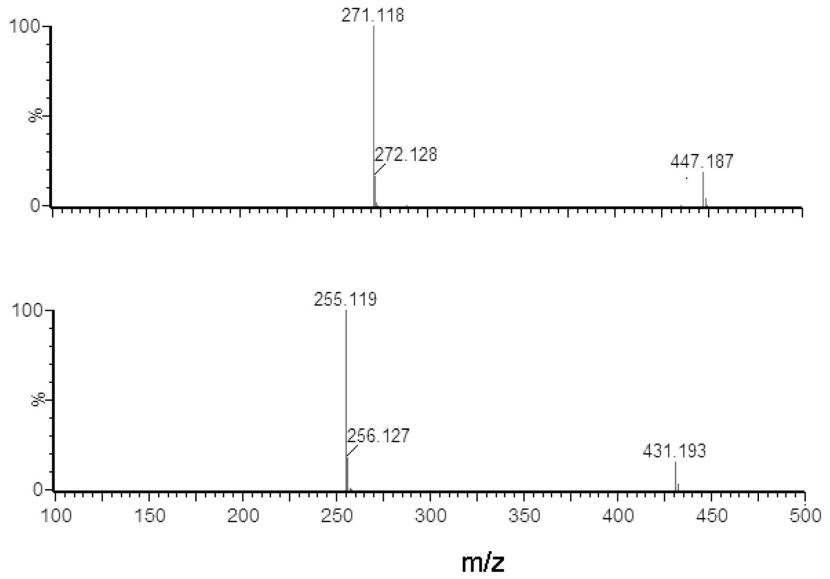

Figure 4. MS-MS spectra of (a) genistein glucuronide (peak 12.43 min from analytical HPLC) and (b) daidzein glucuronide (peak 10.465 min from analytical HPLC) produced by CID experiments.

Table 2. Antioxidant activity of some isoflavones and isoflavone metabolites

Isoflavones	TEAC[a)]	FRAP[b)]
Genistein	0.49 ± 0.02	0.53 ± 0.05
Genistin	0.45 ± 0.05	0.25 ± 0.03
G7G	0.22 ± 0.01	0.27 ± 0.02
Daidzein	0.42 ± 0.03	0.39 ± 0.05
Daidzin	0.34 ± 0.03	0.38 ± 0.04
D7G	0.22 ± 0.02	0.30 ± 0.02

a) TEAC was measured by the scavenging of ABTS$^{\bullet}$ radical.
b) FRAP was measured by the reduction of Fe(III)–TPTZ complex. Data represents Mean ± SD (*n* 3 3) as Trolox or Fe(II) molar equivalent in activity.

numerous studies point to the B-ring phenolic C4′-OH group in isoflavones contributing to the bulk of the antioxidant activity [20, 40]. As expected, G7G and D7G retained free radical scavenging capacity as these metabolites contain B-ring phenolic hydrogen. The TEAC values of genistein (0.49) and daidzein (0.42) were about half of Trolox, in agreement with some recent reports [41, 42]. Both G7G (0.22) and D7G (0.22) have about half the TEAC values of their aglycones, a trend that was also observed for daidzin (0.34) but not genistin (0.45). It is not clear why this is so, but this behaviour that shows little difference in TEAC between genistein and genistin was also repeated in lipid oxidation experiments (see Section 3.5).

3.4 Reducing capacity

The reducing capacity of G7G and D7G were measured using the FRAP assay (Table 2) and were also compared with other isoflavones. This assay measures an antioxidant capacity to reduce Fe(III)–TPTZ to Fe(II)–TPTZ in a coupled redox reaction involving direct electron transfer. This could only come about if the isoflavones were weaker reducing agents than the Fe(III)–TPTZ complex. The FRAP values for genistein and daidzein, expressed as molar equivalent of Fe(II), were 0.53 and 0.39, respectively. Genistin (0.25) and G7G (0.27) have lower FRAP values than genistein, while the FRAP values of daidzin (0.38) and D7G (0.30) were decreased less compared to daidzein.

3.5 Effect of genistein- and daidzein-7-*O*-β-D-glucuronide on the lag phase of lipid oxidation of LDL

While the TEAC and FRAP assays measure antioxidant activity in terms radical scavenging and reducing capacity, such chemical assays are nonetheless indirect methods. The ability of an antioxidant to inhibit lipid oxidation in a liposome system or LDL particle constitutes a direct method of assessing the antioxidative ability of an antioxidant in arresting a biologically relevant oxidative process [43]. The influence of antioxidants on LDL oxidation is an important antiatherogenic property because of considerable evidence that such oxidation is a key process in the disease development [44]. In addition, copper-mediated LDL oxidation represents an important experimental model of atherosclerosis because of the metal association with atherosclerotic lesions [45]. The average human plasma LDL particle contains 1280 molecules of lipids, comprising 86% linoleic acid, 12% arachidonic acid and 2% docosahexaenoic acid [46]. LDL isolated from plasma contains a small quantity of lipid hydroperoxide which can participates in a redox cycling reaction with Cu(II) ions. This reaction generates lipid alkoxyl radical that feed the propagation phase of the lipid oxidation chain reaction, during which conjugated diene reaction products accumulate and proceeds until all

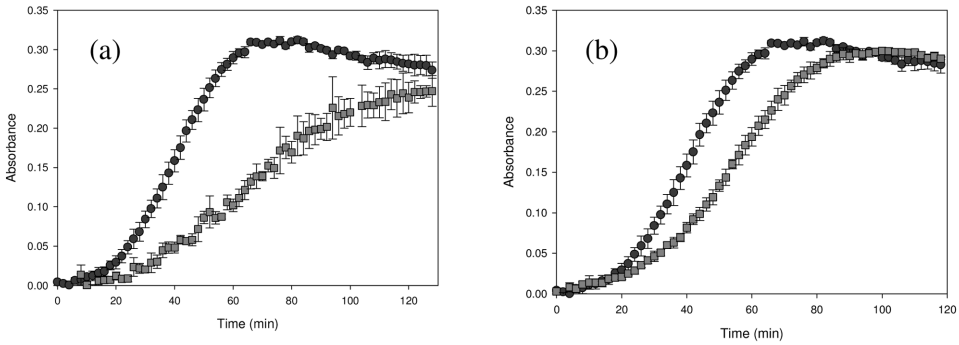

Figure 5. Oxidation of LDL by Cu(II) ions (1 μM) in the absence (O) or presence (□) of (a) genistein-7-*O*-β-D-glucuronide (5 μM) and (b) daidzein-7-*O*-β-D-glucuronide (5 μM).

Table 3. Effects of isoflavones at 5 μM on the duration of lag phase and propagation rate on Cu(II)-mediated LDL oxidation

Isoflavones	Lag time (min) ± SD	Lag phase (%)	Mean propagation rate (μM/min)
None (control)	18 ± 3	100	0.254
Genistein	48 ± 5*	267	0.118
Genistin	44 ± 3	244	0.118
G7G	32 ± 5*	178	0.135
Daidzein	34 ± 1**	189	0.165
Daidzin	28 ± 1	156	0.203
D7G	28 ± 4**	156	0.218

Data represents Mean ± SD ($n \geq 3$).
*, ** ANOVA between pair of values, $p < 0.05$

lipid substrate are consumed [47]. The kinetics of the oxidation can be assessed by continuous monitoring of conjugated dienes formation at an absorbance of 243 nm wavelength. The profile of such a reaction kinetic typically shows an initiation and a propagation phase, which allows us to determine the lag time (from the intersection of the tangents of the two phases) prior to and the rate of oxidation (from the gradient) during the propagation phase (Fig. 2) [50].

The abilities of G7G and D7G to inhibit lipid oxidation of LDL were measured along with their aglycones, genistein and daidzein, and the naturally occurring *O*-glucosides, genistin and daidzin, for comparison. Example kinetics profiles of lipid oxidation of LDL in the presence of G7G and D7G are shown in Fig. 5.

Genistein inhibited lipid oxidation by delaying lag phase by 267% compared to control (100%) incubation without isoflavone under our experimental conditions (Table 3). Genistin, which carries a C7-*O*-glucose unit, has minimal impact on this antioxidant activity in agreement with previous reports [17, 48], which produced a 244% delay in lag phase compared to control. However, substitution of the same hydroxyl group with a glucuronic acid (G7G) resulted in a marked decreased in antioxidant activity (178% delays in the lag phase). This could be a reflection of an increase in polarity that affects partitioning into LDL lipid phase.

Daidzein also inhibited lipid oxidation, but was less effective than genistein, with the lag phase delayed by 189% compared to control (Table 3 and Fig. 5). Daidzin, which contain a C7-*O*-glucose, showed a significantly less pronounced antioxidant activity (156% delays in lag phase). Again, these observations were in agreement with a previous report [17]. It is not clear why this behaviour was not observed for the genistein/genistin pair (above). As with G7G, D7G was less effective than its aglycone, daidzein, but was equally effective as daidzin (156% delays in lag phase) in inhibiting lipid oxidation. It is worth noting that flavonol glycosides were also less active than their aglycones in the inhibition of lipid oxidation in micelles [49], human LDL [50] and red blood cells [51], and against free radical-induced oxidative haemolysis in red blood cells [52, 53].

Two possible mechanisms of action can be envisaged. Isoflavones could inhibit Cu(II)-mediated lipid oxidation of LDL by acting as a metal chelator in the aqueous phase. Genistein, genistin and G7G could conceivably chelate metal ions *via* the C5-OH and C4-keto groups, but this interaction is known to be unfavourable towards Cu(II) ions as indicated by the absence of spectral changes and prooxidant activity in the presence of the ions [54 – 56]. Orthodihydroxyl as present in catechins and C4′-OH as present in resveratrol groups are known to reduce Cu(II) readily [57], but not the C4′-OH in genistein [58]. Based on their structures, daidzein, daidzin and D7G would have no metal chelating capacity and, in common with genistein, their C4′-OH would not be able to reduce Cu(II) ions. But they are still capable of inhibiting lipid oxidation. Therefore, it is more likely that all these isoflavones inhibited lipid oxidation in the lipid phase and presumably acted as chain terminating antioxidants by scavenging propagating radicals. The ability to partition into the lipid phase contributes to a significant degree in an antioxidant ability to inhibit lipid oxidation [59]. Our results indicated that G7G and D7G still retain significant radical scavenging and reducing ability for such antioxidant action as indicated by their significant TEAC and FRAP values, as these metabolites contain unmodified B-ring phenolic hydroxyl group.

The effect of C7 glucuronidation of genistein and daidzein on lipid oxidation of LDL was similar to that of sulphation. Genistein-7-*O*-sulphate and daidzein-7-*O*-sulphate metabolites were also reported to be less active then their aglycones in increasing lag phase of lipid oxidation of

LDL, and that the 4-*O*-sulphate was less active still, while the 4,7-*O*-disulphate was inactive [24]. A similar pattern of inhibition was also observed for the methylated forms where biochanin A (genistein-4′-*O*-methyl) and formononetin (daidzein-4′-*O*-methyl) were less active than their aglycones, while prunetin (genistein-7-*O*-methyl) retain most of the aglycone activity [24]. These results show the importance of the B-ring C4′-OH role in antioxidant activity.

3.6 Effect of genistein- and daidzein-7-*O*-β-D-glucuronide on the propagation rate of lipid oxidation of LDL

The results presented in Table 3 show that the isoflavones genistein, genistin, daidzein and daidzin affected lipids oxidation during the propagation phase, as were reported before [17, 22]. G7G and D7G were found to retain this ability. It showed up in the kinetics profile as a decrease in the rate of lipid oxidation as indicated by the shallower gradient of the propagation phase, with G7G and D7G decreased oxidation rate to 53 and 86% of control without isoflavones, respectively (Fig. 5). Since chain reactions occur in the lipid phase and antioxidants can only affect the reaction in this phase, the fact that the rate of oxidation was decreased would indicate replenishing of lipid phase antioxidants during the oxidation period. Thus G7G and D7G, in common with other isoflavones, are behaving as amphiphilic antioxidants [30, 60]. This contrast with endogenous LDL antioxidant such as α-tocopherol that once 'consumed' can no longer protect further oxidation reactions.

4 Concluding remarks

We presented evidence that G7G and D7G, the main *in vivo* metabolites from soy consumption, retained the ability to inhibit Cu(II)-mediated lipid oxidation of human low density lipoprotein. Nonetheless, the antioxidant activities of the glucuronides were diminished compared to their aglycones. This could be due to a combination of decreased TEAC and FRAP capacities and decreased partitioning into the lipid phase of the LDL particles due to the rather polar glucuronic acid moiety, which is ionised at pH 7.4. Since the isoflavones are less potent antioxidants than the other major flavonoid groups, it diminishes their importance as a source of nutritional antioxidants. But, from the point of view of safety based on our increasing awareness of the potential harmful effect of prooxidant activity possessed by flavonoids which contain the catechol structure, such as some flavonols and the catechins [54 – 56], it would favour compounds lacking prooxidant activity even though their antioxidant activity is low.

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